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Full Length Article

Biodiesel synthesis assisted by ultrasonication using engineered thermo-stable *Proteus vulgaris* lipase

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HIGHLIGHTS

- *Proteus vulgaris* lipase was engineered by disulfide bond introduction.
- Lipase was physically immobilized and entrapped on hydrophobic PS beads.
- These biocatalyst were utilized in the production of biodiesel using non-edible oil.
- Ultrasonication was used to improve the efficiency of lipase catalysed transesterification.
- With sonication 98% yield was obtained in 30 min.

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ABSTRACT

Nature has evolved and designed enzymes to perform an exquisite array of tasks, but in the pursuit of biotechnological interests, these enzymes must often be improved, altered, or even completely redesigned. In the present work, production of biodiesel was carried out using Neem oil and methanol catalysed by "engineered" *Proteus vulgaris* lipase (PVL). Two major issues have been addressed in this study in order to improve the efficiency of biodiesel synthesis by enzyme catalysis. The thermal stability of PVL was increased by introduction of a disulfide bond in G181 and T238 by mutation to cysteines. The transesterification reaction was carried out using sonication under different ultrasonic experimental conditions using a 20 kHz horn. The results showed that the application of ultrasound, using 20 kHz horn with 1 cm tip diameter, decreased the reaction time from 22–24 h to 30 min at an applied power of 40 W and methanol to oil molar ratio of 5:1. Temperature raised due to sonication had no effect on engineered PVL ($PVL^{C181\text{-}238}$) activity. A comparative study of wild type (WT-PVL) and engineered $PVL^{C181\text{-}238}$ for different temperature has been performed and results showed that introduction of a single disulfide in PVL significantly stabilized it, increasing the half-inactivation temperature (IT1/2) from 37 °C for the WT-PVL to 50 °C for the $PVL^{C181\text{-}238}$ engineered one. In biodiesel synthesis also after immobilization on (Polysulfone) PS beads, $PVL^{C181\text{-}238}$ showed better performance compared to WT-PVL.

1. Introduction

Enzyme technology is one of the corner stones of industrial biotechnology. Innovative enzyme solutions can potentially improve efficient biomass utilization, and hence significantly contribute to the transition to a bio-based economy by offering environmentally friendly technologies for the production of biofuels, chemicals, and materials from biomass resources. Increased energy demands have turned scientist's interest in search of alternative fuels from renewable sources, as they are biodegradable and lesser toxic. Biodiesel (mono alkyl esters of long-chain fatty acids) is receiving increasing global attention as an attractive replacement for fossil fuel due to its favourable properties like derivation from locally available renewable biological resources, environmental friendly due to reduced emission & self-subsistent energy source. Although biodiesel is presently being synthesized in many countries, the high cost of production techniques along with the major production from edible oils are the major obstacles for its further development and wide application [1]. Methods of biodiesel synthesis from fats & oils can be grouped into base/acid & enzyme-catalyzed transesterifications [2–4]. The base/acid catalyzed methods are most industrially used but have disadvantage of pre-treatment of oil having high water & FFA, soap forma-

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tion, wastewater treatment, difficult separation of biodiesel & glycerol, catalyst removal compared to enzymatic transesterification [5,6]. Synthesis of biofuel requires efficient methodology with a fewer steps to be economically and environmentally feasible. Lipases have found a wide range of applications, ranging from bioremediation, biosensor development, bioconversion to biofuels and other biotechnologically important value-added products so; the emphasis has therefore been placed on these enzymes and their applications. The lipase catalysed esterification has been considered as friendly and thus "green" for the environment.

Although lipases derived from natural sources can be used in biodiesel production, they typically lack the desirable features that are suitable for industrial scale reactions. Specifically, natural lipases have maximum catalytic activities in the temperature range 30–50 °C. At these temperatures, the transesterification reaction has low reaction rate which makes the process time-consuming and less economically competitive [7]. Increasing the working temperature range of lipases by improving thermal stability, therefore, it is a critical aspect of lipase engineering. The current performance of lipases, however, still falls short of industrial expectations in terms of long-term thermostability.

In the present work lipase from *Proteus vulgaris* was explored. *Proteus vulgaris* belongs to the Proteus/psychrophilic subfamily of I.1 lipases and lack a leader sequence and a disulfide bond present in other family I.1 and I.2 lipases [8]. The lipase from *Proteus vulgaris* is tolerant to short-chain alcohols (MeOH and EtOH) and synthesizes fatty acid methyl esters (biodiesel) even in the presence of high concentration of water [9]. To date, there are no reports of the reengineering of *Proteus vulgaris* lipase. Thermo-stability is an important property that contributes to the industrial usefulness of an enzyme. The introduction of disulfide bonds is a common strategy to improve enzyme stability that has successfully been applied to other lipases [10]. One of the common drawbacks with the use of enzyme-based processes is the high cost of the enzyme. After immobilization of enzymes, if they can be used repeatedly would bring the cost down and apart from this they have superior thermal and operational stability through a range of pH values and ionic concentrations and are more resistant to denaturation than their native soluble forms [11–18].

Several studies have used acoustic cavitation and hydrodynamic cavitation to overcome the mass-transfer limitation problem in biodiesel synthesis [19–26]. The chemical and physical effects of ultrasound arise from the acoustic cavitation which produces extreme conditions locally such as high temperature, high pressure, acoustic microstreaming, turbulence, and high shear forces that can help to generate fine emulsions between immiscible fluids to enhance mass transfer and hence the rate of transesterification reaction [27].

The present work was aimed at lipase catalysis in combination with enzyme engineering for the efficient creation of novel and practical biocatalysts (with improved catalytic performance, thermo-stability) for cost effective, high yield enzymatic esterification techniques using immobilized enzyme system for synthesis of biodiesel. Ultrasonic irradiation was used to enhance the efficiency of biodiesel production process. The target was to exploit oil from non-feed crops, which is a significant step as the world is facing the energy crisis that disrupts the economic stability & threatens global security.

2. Methods

2.1. Materials

Commercially available neem (*Azadirachta Indica*) oil was used. Lipase genes from *Proteus vulgaris* are purchased from genescript and a standard fatty acid methyl ester (FAME) mix (chromatographically pure) as a reference were purchased from Sigma-Aldrich. Primers for cloning and mutagenesis were designed and got from Sterling Biologicals. Escherichia coli (DH5Alpa) and vector pIQ- MBP- SacB preserved in laboratory were used for gene cloning, and E. coli BL 21 (DE3) also preserved in laboratory were used for expression. E. coli DH5 α and BL 21 (DE3) were cultured at 37 °C in Luria–Bertani medium containing 100 μg/mL Ampicillin for selection. HPLC grade acetonitrile (99.7%) and hexane (99%) were purchased from Ajax Finechem. Methanol (99.7%) was purchased from Scharlau, di-sodium hydrogen orthophosphate (98%) and sodium dihydrogen orthophosphate (99%) were purchased from Chem-Supply. Miniprep kits were used from bioline. Polysulfone polymer (Udel P-3500, Solvay Advanced Polymer, India and dimethyl formamide (DMF) (Qualigen, India) were used for beads preparation. Folin reagent (SD fine Chem., India), di-sodium tartarate (SD fine Chem., India), BSA fraction V (Sigma-Aldrich, USA) were used for protein estimation. Acacia powder (SD fine Chem, India) was procured. All solutions were prepared in high purity water extracted from a Millipore system.

2.2. Disulfide bond introduction

Pseudomonas aeruginosa and Burkholderia cepacia are homologous lipases (42% and 38% ID to PVL, respectively) and they have a single disulfide bond between residues 181 and 238. Due to the structural conservation of this region and the proximity of G181 and T238 in PVL, a disulfide bond was introduced between residues G181 and T238 by mutation to cysteines.

2.3. Cloning of wild type and disulfide introduced lipase gene

The wild-type and disulfide introduced lipase gene from *Proteus vulgaris* having BamHI and HindIII sites was cloned into chemically competent DH 5 alpha cells. Lipase gene and the vector pIQ- MBP-SacB were digested with BamHI and HindIII and recovered through agarose gel electrophoresis, and then ligated by T4 DNA ligase having the corresponding sticky ends. The ligated plasmid pIQ- MBP-SacB -lip was transformed into E. coli DH 5 alpha using heat shock method. A single colony of the transformant was selected and transferred into 5 mL LB broth with 100 μg/mL ampicillin and incubated overnight with vigorous shaking (200 rpm) at 37 °C. The culture was used to extract recombined plasmid through alkaline lysis procedure.

2.4. Expression and purification of WT and mutant $PVL^{C181\text{-}238}$

Over-expression of WT-PVL and mutant $PVL^{C181\text{-}238}$ was carried out in E. coli BL 21 (DE3) (Agilent). Single transformants were transferred to 2 L of Luria-Burtani (LB) media containing ampicillin for incubation at 37 °C and grown to an OD 600 of 0.6. Protein expression was induced by 0.5 mM IPTG at 16 °C for 16 h. Cells were harvested by centrifugation at 4 °C for 5 min and purified by Talon IMAC chromatography. The cells were lysed by 0.1 mg/mL lysozyme, and by sonication using 10 s on & 10 s off for 15× cycles. Then the supernatant (soluble protein) was harvested by centrifugation at 10,000×*g* at 4 °C for 20 min. The supernatant (crude enzymes) was removed and directly mixed with 1-mL Talon resin & incubated for 1 h at 4 °C with gentle mixing. Then prepared an empty column PD10 and added the talon/supernatant to the column & subsequently eluted with an elution buffer (300 mM NaCl, 50 mM sodium phosphate, and 250 mM imidazole, pH 8.0). Wild-type and mutant PVLs were then dialyzed into 20 mM Tris–HCl pH 7.5 containing 100 mM NaCl and flash frozen as droplets in liquid N_2 prior to storage at −80 °C.